

Characterization and mapping of QTL used in breeding of Scots pine (*Pinus sylvestris* L.)

Aleksandra Nowicka¹, Joanna Ukalska², Joanna Simińska³, Iwona Szyp-Borowska⁴ ✉

¹ Polish Academy of Sciences, Institute of Fundamental Technological Research, Pawińskiego 5b, 02-106 Warsaw, Poland

² Warsaw University of Life Sciences, Faculty of Applied Informatics and Mathematics, Nowoursynowska 159, 02-776 Warsaw, Poland

³ Warsaw University of Life Sciences, Faculty of Agriculture and Biology, Nowoursynowska 159, 02-776 Warsaw, Poland

⁴ Forest Research Institute, Department of Silviculture and Genetics, Sękocin Stary, Braci Leśnej 3, 05-090 Raszyn, Poland, phone: +48 22 7150481, fax: +48 22 7150313, e-mail: I.Szyp@ibles.waw.pl

ABSTRACT

This paper reports the construction a map based on Amplified Fragment Length Polymorphic DNA (AFLP) in Scots pine (*Pinus sylvestris* L.). The main purpose of map construction was its application to quantitative traits loci (QTL) mapping for breeding traits economically important in Scots pine breeding program such as tree height and diameter at breast height, number of needles and their length, width, and area. Genomic DNA of needles and haploid megagametophytes from seeds originating from a single tree were amplified with 25 AFLP primer-enzyme combinations with three or four selective nucleotides. Sixteen of them generated easily readable patterns and revealed a polymorphism. Each analyzed marker was tested for the expected 1 : 1 segregation ratio using χ^2 – test and only 6 were significant with ($\alpha \leq 0.05$). The total map size equaled 291,7 cM and all markers were distributed within one linkage group. For all traits only one QTL associated with tree height (H) was detected.

KEY WORDS

QTL, *Pinus sylvestris*, genetic mapping, AFLP

INTRODUCTION

Plant breeding is a dynamic area of applied science, which relies on genetic variation and selection and leads to improved plants for traits and characteristics that are of interest for the user. Most characters important to forestry, such as biomass production, wood quality, biotic and abiotic stress response, are complex quantitative traits (Plomion et al. 2003). Tree improvement is based on tra-

ditional breeding techniques, that is selection of superior trees for volume and stem straightness, grafting these into breeding orchards and producing seed orchards. The key difficulty with traditional approaches in tree breeding is the long growth cycles, which make this process very time-consuming. Many traits of interest, such as wood properties, change during growth and maturation. Another trouble is a low heritability for many traits of practical interest in forest trees. Moreover, selection for

one chosen feature may lead to a change of another one. In the context of these problems, any other tool which can accelerate the selection process and enhance the productivity of the traditional approach is of significant value. For that reason, quantitative traits loci (QTL) mapping is very important trend of forest trees genetics. Mapping of the genome regions containing or linked to the genes that control quantitative traits is done using molecular tags such as Amplified Fragment Length Polymorphism (AFLP). This is an early step in identifying and sequencing the actual genes underlying trait variation.

In conifers such as loblolly pine (*Pinus taeda* L.), maritime pine (*Pinus pinaster* Ait.), Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) and sugi (*Cryptomeria japonica* D. Don), the main focus of QTL studies has been on economical and adaptive traits such as wood properties (Groover et al. 1994; Kuramoto et al. 2000; Sewell et al. 2000; Sewell et al. 2002; Brendel et al. 2002; Brown et al. 2003; Pot et al. 2006), and growth characteristics (Yoshimaru et al. 1998; Ukrainetz et al. 2008; Pelgas et al. 2011). Up to now, few QTL studies have focused on adaptive characters related to phenological traits and cold hardiness (Jermstad et al. 2001a,b; Jermstad et al. 2003; Wheeler et al. 2005). These studies provide an essential understanding of the structure of genome fraction related to bud flush, bud set, and height growth in conifer species, and allow an indirect selection process where a trait of interest is selected, not based on the trait itself, but on a marker linked to it (Marker-assisted selection – MAS).

Scots pine is the most important forest forming species in Poland. Therefore, choice of this species for analysis of genetic control of economically important traits based on QTL mapping is expedient.

Larceteau et al. (2000) presented preliminary research concerning detection QTL for economically important traits in *Pinus sylvestris*. Several studies have been completed to identify QTLs for height (Larceteau et al., 2000, 2001) or growth parameters in Scots pine (Sillanpää et al. 2011). Presently, nearly complete genetic map of *Pinus sylvestris* was constructed using AFLP markers based on two-way pseudotestcross strategy in a full-sib family. The genetic map covers 98% of genome with a framework marker interval of 20cM for both parents (Yin et al. 2003).

Mapping of QTLs by means of molecular markers and improving important traits are key purpose in

breeding programs. MAS (marker-assisted selection) has potential to make traditional breeding strategies more efficient. By using molecular markers closely linked to, or located within, one or more QTL, information at DNA-level can be used for early estimate and selection of valuable genotype. The potential benefits of MAS are greatest for traits that are difficult, time-consuming or expensive in measuring. The example of wood density in *Pinus taeda* shows usefulness of this method (Groover et al. 1994). The potentiality of identification of loci controlling wood density could enable to determine a tendency of individuals for production of specific density wood on seedling stage, before final disclosure this trait.

The aim of this study was detection of genetic linkages between molecular markers and loci involved in the expression of quantitative traits, economically important in Scots pine breeding program such as tree height and diameter at breast height, number of needles and their length, width, and area. Characterization of inheritance of these traits is important to ensure long-term sustainability of forest resources and their production.

MATERIAL AND METHODS

Plant material

The mapping population consisted of the offspring of the elite tree number 167 of *Pinus sylvestris* derived from open pollination. Seed orchard was established in 1990 in Skierniewice, Forestry Ryłsk. Needles and open pollinated seeds were collected from 120 progenies. Seeds were germinated on wet filter papers for seven days. The haploid megagametophyte (nutritive tissue surrounding the embryo originated from the female megaspore) of each F₂ was removed during germination and stored at – 80°C before DNA extraction.

Phenotypic measurements

Total tree height and trunk diameter at breast height i.e. 1,3 m from ground level were measured in winter 2006. The number of needles was assessed on a length of 10 cm from the apical bud. Characteristics such as the length of the needles their width, and area were measured after scanning using the computer program *digiShape* (Moraczewski 2005).

Linkage analysis and map construction

Total genomic DNA was isolated from fresh needles and megagametophytes using a DNeasy Plant QIAGEN Kits. Restriction digests and ligation were performed using the AFLP Core Reagent Kit (Invitrogen, Life Technologies). Preamplification was carried out with standard *EcoRI* (E) and *MseI* (M) adaptors with one additional nucleotide. Selective amplification was carried out with 25 primer-enzyme combinations with three or four selective nucleotides (tab. 1). All PCR conditions were as described by Costa et al. (2000). Selected DNA fragments were identified by electrophoresis in a 5% denaturing polyacrylamide gel. Electrophoresis was performed –using the Sequi-Gen Nucleic Acid ® GR Electrophoresis Cell / PowerPac 3000 System (Bio-Rad) in 5 × TBE (Tris/Borate/EDTA) for approximately 2.5 h (60 W, 400 mA, 1400 V).

Tab. 1. Combinations of primers pairs used in the selective amplification with E – *EcoRI* adapter – 5'-GACTGCGTACCAATTC-3', M – *MseI* adapter – 5'-GATGAGTCCTGAG-TAA-3', * primer-enzyme combinations generated easily readable patterns and revealed a polymorphisms

Primer ID	<i>EcoRI</i>	<i>MseI</i>
1	2	3
E1/M3	E-ACAA	M-CTC
E1/M4*	E-ACAA	M-CCTC
E1/M5	E-ACAA	M-CCGC
E2/M3	E-AGG	M-CTC
E2/M4	E-AGG	M-CCTC
E2/M5	E-AGG	M-CCGC
E2/M6	E-AGG	M-CAG
E2/M10	E-AGG	M-CTG
E3/M3*	E-ACAT	M-CTC
E3/M4*	E-ACAT	M-CCTC
E3/M5*	E-ACAT	M-CCGC
E4/M3*	E-ACA	M-CTC
E4/M4*	E-ACA	M-CCTC
E4/M5	E-ACA	M-CCGC
E5/M3*	E-ACG	M-CTC
E5/M6*	E-ACG	M-CAG
E5/M7*	E-ACG	M-CTA
E5/M8*	E-ACG	M-CAC
E5/M10*	E-ACG	M-CTG

1	2	3
E6/M8*	E-ACGC	M-CAC
E6/M9*	E-ACGC	M-CAT
E9/M3	E-AGC	M-CTC
E9/M6*	E-AGC	M-CAG
E9/M8*	E-AGC	M-CAC
E9/M10*	E-AGC	M-CTG

Detection of the products was conducted using silver staining kit from Promega according to the procedure specified by the manufacturer. Chi-square tests were performed to check if individual fragments segregated in the Mendelian segregation ratio 1 : 1. For map construction, the mapping program MAPMAKER/EXE vers. 3.0 was applied. The linkage map for progeny was used for the identification of genomic regions controlling height growth, diameter and traits for needles characteristic. QTLs were mapped using interval mapping method and linear regression with Windows QTL Cartographer 2.5 (Department of Statistics, North Carolina State University, Raleigh, NC; <http://statgen.ncsu.edu/qtlcart/WQTLCart.htm>). In the case of haploid megagametophyte, the F2 backcross option was used. Linkage groups were assigned with thresholds set at a LOD score of 3.0.

RESULTS

Phenotypic measurements

For the progeny of *Pinus sylvestris* L. we analyzed six characteristics: 1) diameter at breast height (DBH), 2) height of the tree (H), 3) the number of needles per 10 cm shoot from the apical bud, 4) needle width, 5) needle length, 6) and needle area (tab. 2). Results of statistical analyzes indicate small variation for all studied traits. The lowest variability (to 15%) was found in the case of the width of needles, tree height (H), the length of the needle and the number of needles on the shoot. More than 16% of variability was observed for DBH. The average size of needles was 273,36 mm and the coefficient of variation was 18,85%. It is the most variable trait. The normality of the distributions of the traits was evaluated using the Kolmogorov–Smirnov and Shapiro–Wilk tests, and all of them showed normal distribution.

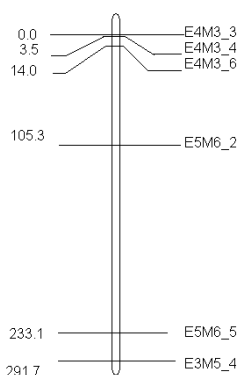
Tab. 2. Standard values for the morphological features of the population of *Pinus sylvestris* L.

Feature	N	\bar{x}	Min value	Max value	Standard deviation	Median	CV (%)
1	124	19,40	13,50	28,60	31,89	19,4	16,38
2	124	929,50	610,00	1220,00	111,69	935,0	12,01
3	124	80,70	54,60	114,60	11,54	80,2	14,30
4	124	2,02	1,60	2,48	0,17	2,0	8,56
5	124	61,79	38,96	82,12	8,51	61,5	13,78
6	124	273,36	133,88	398,46	51,52	269,0	18,85

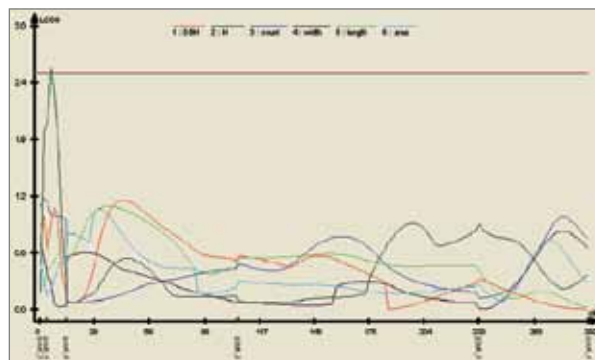
N – number of samples; CV – coefficient of variation.

Linkage analysis and map construction

The genome of conifers are very large. Consequently, the normal AFLP selective amplifications using E+3/M+3 primer combinations resulted in too many faint and overlapping fragments. To solve this problem we added a fourth selective nucleotide to the M and E primers. We used 25 primer combinations for screening the mapping population and to construct an AFLP map. Sixteen of them generated easily readable patterns and revealed a polymorphism (tab. 1). The number of polymorphic fragments per primer-enzyme combination ranged from 2 to 4 with sizes between 145 bp and 700 bp. Each analyzed marker was tested for the expected 1 : 1 segregation ratio using χ^2 -test and only 6 were significant with ($\alpha \leq 0.05$). The total map size equaled 291,7 cM and all markers were distributed within one linkage group (fig. 1).

**Fig. 1.** Linkage map of *Pinus sylvestris* based on AFLP markers. Marker names containing the primer ID are given on the right of the map and centimorgan distance on the left

For all traits only one QTL associated with tree height (H) was detected (fig. 2). The graphic shows the markers on the chromosome and their distances. The analyzes revealed the presence of QTL associated with the height of a tree. The QTL is located between markers E4/M3_4 and E4/M3_6.

**Fig. 2.** Calculated values of LOD along the entire length of the chromosome. Occurrence of the peak LOD curve exceeds a critical value determines the space where QTL associated with a given trait it is located

DISCUSSION

In this paper we searched QTLs related to traits of important economic value. For all traits only one QTL associated with tree height H was detected. Our results are also consistent with previous studies on identification markers associated with economically important traits of *Pinus sylvestris*, using BSA approach (Bulk Segregant Analysis) (Szyp-Borowska et al. 2011). In this study one locus was detected, also linked to height. It is suggested that tree height may be controlled by genes with major effects. Height growth was the trait most often studied, but in most of this studies, zero to three QTLs were found, accounting for 10–25,9% of phenotypic variation (Van Buijtenen 2001). According to Hannrup et al. (2000) the height of tree is less strongly influenced by environment than number of needles and their length, width, and area, so the possibility of finding QTLs linked to this is higher.

Most of the mapping in plants is carried out by crossing pure lines of crop species and then studying recombination in progeny i.e. F2 or BC generations. This means that it is possible to map only those loci which differ between the two lines. Another situation is faced in heterozygous and outcrossing trees. In this study the haploid

megagametophyte segregation of individual trees was used as mapping population. In conifers, haploid megagametophytes allowed direct analysis of linkage and construction of genetic maps (Hurme et al. 2000).

The main purpose of map construction must be its application to QTL mapping for breeding traits. The total height QTL might be a very useful characteristic for MAS.

Screening the primers for many reproducible markers is a prerequisite for any successful AFLP analysis. Our results suggest that larger genomes require greater numbers of nucleotides to reduce the number of AFLP fragments to a scorable number per PCR. We tested the properties of AFLPs in two ways. First we used the standard protocol for complex genome analysis (preamplification +1 selective base primers and amplification with +3 primers) and secondly we modified the protocol by replacing E+3 primer with E+4 primers and M+3 primer with M+4. Both +3 and +4 was informative but the modification appeared to reduce the number of bands. The same remarks were made by Cato et al. (1999) and Cervera et al. (2000).

The results reported in this study are preliminary. Mapping additional markers may fill the large gaps in the map. It should also be stressed that the 120 samples analysed in this study did not represent a reliable sample. The small number of markers segregating in 1:1 ratio might be one reason why we received only one linkage group. Further, a more detailed analysis on QTL locations between the genetic maps for *P. sylvestris* presented here and maps which were constructed already based on the same set of markers and their relation to the another references maps will be also a next task.

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